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Silica microspheres are superior to polystyrene for microvesicle analysis by flow cytometry ☆

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ABSTRACT

Background: Cell-derived microvesicles (MVs) in biological fluids are studied for their potential role in pathological conditions. Flow cytometry is used to characterize MVs. Polystyrene microspheres are often used in flow cytometry to distinguish MV from cells by setting a 1-μm MV gate in a side-scatter (SSC) vs. forward-scatter (FSC) dot plot. Polystyrene microspheres, however, exhibit higher FSC and SSC than MVs of equal size. Consequently, some platelets are included within the MV gate, which incorrectly increases the reported percentage of platelet-derived MVs. Silica microspheres exhibit FSC that is closer to that of cellular vesicles and, therefore, should permit more accurate discrimination of MV from platelets.
Objective: Compare silica with polystyrene microspheres to calibrate flow cytometers for definition of MV population and estimation of MV sizes.
Methods: Silica and polystyrene microspheres of various sizes were used in flow cytometry assays to define MV populations and determine platelet and MV sizes in human plasma samples. Sizes determined by flow cytometry were compared to sizes determined by resistive pulse sensing (RPS) method.
Results/Conclusion: Use of 1.0-μm polystyrene microspheres to define the upper MV gate produced a median platelet contamination of 16.53% (8.24, 20.98) of the MV population; whereas, use of 1.0-μm silica microspheres excluded platelet events completely. Calibration with silica microspheres resulted in significantly better estimation of MV diameter than calibration with polystyrene microspheres. We conclude that silica microspheres are superior to polystyrene microspheres as standards to define MV populations without platelet contamination and to determine MV sizes by flow cytometry for a given cytometer.

Introduction

Cell-derived microvesicles (MVs) are submicron vesicles released from activated, apoptotic, or injured cells [1,2]. Microvesicles derived from platelets, leukocytes, and endothelial cells have been reported in a variety of biological fluids that include plasma, cerebral spinal fluid, and alveolar lavage fluid and have been associated with several pathological conditions that include thrombosis, diabetes, myocardial infarction, traumatic brain injury, pulmonary hypertension, brain hemorrhage, and acute respiratory distress syndrome [3–7]. Microvesicles have also been described as mediators of intercellular signaling and transport [8,9].

To gain insight into the various functions of MVs, it is important to accurately characterize their cellular origin, phenotype, concentration, and size. Size distributions of MV populations have recently emerged

as a parameter of interest [10–12]. Microvesicle size and its relation to composition, functional activity, and clinical significance has been reviewed by Jy et al. [13]. In an earlier study, Jy et al. stated that platelet-derived procoagulant activity in thrombotic patients is attributed to larger-size MVs that are > 1.0 micron in size [14]. A study by Dean et al. indicated that different sizes of platelet-derived microvesicles (PMVs) differed significantly in their contents of plasma membrane receptors and adhesion molecules, chemokines, growth factors, and protease inhibitors. In that study, the authors reported four different size ranges of PMVs (separated by gel filtration chromatography), of which the smaller two PMV size ranges inhibited collagen/adenosine-diphosphate-mediated platelet thrombus formation [15]. Therefore, accurate determination of the sizes of MV populations in biological fluids is important in elucidation of MV functions.

Microvesicles in biological fluids can be quantified by several techniques that include resistive pulse sensing (RPS), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and flow cytometry (FCM) [16]. Flow cytometry is the most prevalent method of MV detection and characterization since in addition to concentration, information on the origin, phenotype, and size of MVs can be acquired on thousands of MVs in each sample within a short time. In flow cytometry, MV are identified as events that are ≤1 μm in size as defined by side scatter

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(SSC) and forward scatter (FSC) characteristics of 1-μm polystyrene microspheres used to set the upper limit of the MV SSC vs. FSC gate [17,18]. The lower limit of the MV gate is defined by the smallest polystyrene microsphere FSC that is discernable from noise, which is 400- to 500-nm polystyrene microspheres for many cytometers, while some new generation cytometers equipped with a photomultiplier tube FSC detector (FSC-PMT) and/or wide angle forward scatter detection or with high sensitivity SSC detection system are capable of discerning as low as 160-200-nm polystyrene microspheres from noise [19].

Polystyrene microspheres, however, have a higher refractive index (η_{PS}) than cellular material (η_{cells}) (1.59 vs.1.39, respectively), which causes polystyrene microspheres to scatter more light than cellular vesicles of the same size [19,20]. Consequently, FSC from polystyrene microspheres underestimates MV diameter. Reports have shown that vesicles are 2-3 times larger than polystyrene microspheres with the same FSC depending on the instrument type, optical configuration, and settings (16, 19). Thus, establishing the upper MV gate using 1-μm polystyrene microspheres actually includes vesicles that are within the platelet size range.

Silica microspheres, however, have a lower refractive index ($\eta_{Si} = 1.45$) that is closer to the refractive index of cellular vesicles ($\eta_{cells} = 1.36$-1.42). Therefore, the optical properties of silica microspheres are more similar to the optical properties of vesicles than those of polystyrene microspheres [21]. Consequently, FSC of silica microspheres is closer to FSC of cells and cellular particles. We, therefore, hypothesized that use of FSC from silica microspheres will yield closer estimates of the actual sizes of MV and of the 1-μm upper limit of the MV gate than use of FSC from polystyrene microspheres.

In this study, we demonstrated that calibration of a flow cytometer using silica microspheres of various sizes enables establishment of MV SSC vs FSC gates that exclude platelets; whereas, MV gates established using polystyrene microspheres include platelet-sized events.

Materials and Methods

Reagents and Supplies

Anti-human CD41a labeled with APC-H7 (clone HIP8), citrated Vacutainer® tubes, and 19-gauge needles were obtained from BD Biosciences (San Diego, CA, USA). Hanks' balanced salt solution (HBSS) was obtained from Life Technologies (New York, USA). Polystyrene microspheres were obtained from Bangs Laboratories (Fishers, IN, USA), and silica microspheres were obtained from Polysciences (Warrington, PA, USA). The microsphere standards are traceable to National Institute of Standards and Technology (NIST) Standard Reference Materials.

Plasma Isolation

In accordance with a protocol approved by the institutional review board, a 19-gauge needle was used to collect blood from seven healthy non-fasting volunteers into Vacutainer® tubes (BD Biosciences, San Jose, CA, USA) that contained sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood tube at 200 ×g for 10 min. Platelet-poor plasma (PPP) was prepared by centrifugation of the blood tube at 3,000 ×g for 10 min followed by a second centrifugation of the upper two thirds of the plasma fraction at 3,000 ×g for 10 min in 12 mm x 75 mm polypropylene tubes. The upper two thirds of the plasma were collected carefully using a pipet without disturbing the pellet and transferred to 12 mm x 75 mm tubes for the second centrifugation.

Calibration of Flow Cytometer for Size Determination

Flow cytometric measurements were carried out on a Canto II (BD Biosciences) flow cytometer equipped with a 405-nm laser, a high power (200 mW) 488-nm laser, and a 640-nm laser. Forward and SSC were measured off of the 488-nm laser and the SSC threshold was set to 200. To minimize background noise from small dust particles in the sheath fluid, the two standard in-line 0.2-μm filters on the Canto II were replaced with 0.1-μm filters. To reduce background particles in sample suspensions, HBSS used to resuspend polystyrene and silica microspheres and to dilute PRP and PPP was passed through a Stericup Express Plus 0.1-μm filtration system (Millipore Corp., Billerica, MA) prior to use.

Polystyrene microspheres (390 nm, 505 nm, 794 nm, and 990 nm) and silica microspheres (500 nm, 700 nm, 1000 nm, and 2000 nm) were used to calibrate the Canto II flow cytometer for size determination. Calibrations were performed daily. One drop (~50 μl) of each size of polystyrene microsphere stock from their bottles was diluted with 10 ml of filtered HBSS in separate tubes and mixed thoroughly by vortexing. This was repeated for silica microspheres of different sizes in a separate set of tubes. Specific volumes (5, 25, 50, and 75 μl) of diluted polystyrene microspheres or diluted silica microspheres were added to tubes containing 2 ml of filtered HBSS. A mix of all four sizes of microspheres in 2 ml of filtered HBSS was also prepared from diluted stock suspensions. Forward scatter and SSC data were acquired on individual microsphere suspensions, and a tight gate was drawn around each microsphere population in an SSC vs. FSC plot. After the microsphere gates were defined, data was acquired on the polystyrene and silica microsphere mixtures. Mean FSC value for each microsphere population within that mixture was obtained and logarithmically transformed. Size calibration curves were generated by linear regression analyses of log mean FSC vs. microsphere size data. Following acquisition of FSC and SSC data on both, polystyrene and silica microspheres, FSC and SSC data was acquired on PRP and PPP samples. The cytometer configuration and settings remained the same for microsphere and plasma samples.

Platelet and Platelet MV Identification and Size Determination

To identify platelets, 5 μl of PRP or PPP was mixed with 5 μl of anti-CD41a antibody conjugated with APC-H7 (titrated on platelets) plus 90 μl of 0.1-μm filtered HBSS, and incubated at 4 °C for 30 min in dark followed by 10-fold dilution with 0.1-μm filtered HBSS prior to analysis. Samples were then analyzed on a BD Canto II flow cytometer using a 640-nm laser to measure anti-CD41a-APC-H7 fluorescence and a 488 nm laser to measure FSC and SSC. Twenty thousand platelets were counted for each PRP sample and 100,000 MVs were counted for each PPP sample. Platelets were identified by anti-CD41a-APC-H7 fluorescence vs. FSC density plots (2% probability). Mean platelet size in PRP was calculated from the mean FSC within the platelet gates using each calibration curve. Microvesicle size in PPP between 600 nm and 800 nm was calculated from each calibration curve and from platelet-derived vesicle:microsphere size equivalency ratios (SERs) as described in "Results."

Platelet and MV sizes were also measured by RPS with a qNano particle size analyzer. Resistive pulse sensing measures transient changes in current flow through pores of a membrane as particles pass through, and the magnitude of each transient change is proportional to particle size according to the Coulter principle [16]. The mean size of platelets in each PRP sample determined by flow cytometry using polystyrene and silica calibration curves was compared with mean sizes determined by RPS. Similarly, the mean size of MV in PPP between 600 nm and 800 nm was determined by each method and compared.

Statistical Analyses

Repeated-measures analysis of variance (ANOVA) with Tukey adjustment post hoc was performed to compare platelet sizes measured by flow cytometry (polystyrene vs. silica microspheres) and by RPS.

Analysis of covariance (ANCOVA) was performed to compare the slopes and intercepts.

Results

Side scatter vs. FSC dot plots of polystyrene and silica microspheres of different sizes and size calibration curves established from these dot plots are shown in Fig. 1. Plots A and C show that distinct populations of microspheres were observed for all sizes of polystyrene and silica microspheres. There was no overlap between adjacent sizes ensuring resolution of all sizes. Curves B and D show that Log Mean FSC increased linearly as a function of the size of polystyrene and silica microspheres ($R^2 = 0.985$ and 0.983 for polystyrene and silica microspheres, respectively). The slope of the polystyrene microsphere curve was 1.9 times greater than the slope of the silica microsphere curve (slope of polystyrene vs. silica: $p < 0.001$).

Fig. 2 shows anti-CD41a-APC-H7 fluorescence vs FSC density and dot plots of a representative PRP sample. Gates define the platelet population in each plot based upon anti-CD41a-APC-H7 fluorescence and FSC. The dashed horizontal line labeled in the dot plot identifies the fluorescence threshold for CD41a$^+$ events set using the isotype control. Platelets and MV are identified.

Platelet size determined by flow cytometry calibrated with polystyrene and silica microspheres was compared to platelet size measured by RPS and the results are shown in Fig. 3. Sizes determined by flow cytometry were smaller than those measured by RPS (Fig. 3A). The mean size of platelets in each PRP sample calculated from polystyrene calibration curves was, however, significantly smaller than the mean size of platelets calculated from silica calibration curves (mean platelet size from n = 7 PRP: 1114 vs 1942 nm, polystyrene vs silica microspheres, ($p < 0.0001$). The mean size of platelets measured by RPS was 2299 nm. Fig. 3B shows the ratios of platelet size as measured by RPS to the platelet size determined by flow cytometry using polystyrene and silica microspheres for size calibration. The RPS:flow cytometry platelet size ratios represent vesicle:microsphere size equivalency ratios (SERs), that is, the diameter of vesicles and microspheres with equivalent FSC. Vesicle:microsphere SERs for polystyrene and silica

microsphere calibrations were 2.0:1 and 1.2:1, respectively. Thus, platelet sizes determined from polystyrene calibration curves were on average only half of the sizes determined by RPS; whereas, platelet sizes determined from silica calibration curves averaged 85% of RPS sizes.

To demonstrate the utility of vesicle:microsphere SERs derived from platelets in the determination of MV size, we used the ratios calculated in Fig. 3B to adjust estimations of MV size by flow cytometry as derived from the polystyrene and silica microsphere calibration curves. Fig. 4 depicts a comparison of the mean size of MV between 600 nm and 800 nm as measured by RPS and as determined by flow cytometry with and without adjustment for vesicle:microsphere SERs. To estimate MV size without adjustment, FSC gates for 600 nm and 800 nm were calculated from the silica microsphere calibration equation shown in Fig. 1. The polystyrene and silica microsphere calibration equations in Fig. 1 were then used to calculate mean unadjusted sizes from the mean FSC values between those gates. These sizes are shown in Fig. 4A along with MV sizes measured by RPS. Fig. 4B depicts the comparison of MV sizes by RPS and flow cytometry with adjustment. To adjust the sizes for the FSC differences between microspheres and vesicles of equal size, the 600-nm and 800-nm gate FSC values were adjusted to 300-nm and 400-nm FSC values for polystyrene microspheres and to 500-nm and 667-nm FSC values for silica microspheres through division by vesicle:microsphere SERs of 2.0 and 1.2 for polystyrene and silica, respectively. Forward scatter for the adjusted MV gates was then calculated from polystyrene and silica microsphere calibration equations in Fig. 1. Then, mean MV sizes within these gates were calculated from equations in Fig. 1 and were multiplied by the polystyrene and silica vesicle:microsphere SERs to calculate the actual MV size. Without adjustment, use of the polystyrene microsphere calibration equation significantly underestimated MV diameters as measured by RPS and relative to MV diameters calculated from the silica microsphere calibration equation. Adjusted sizes of MV determined by flow cytometry using either silica or polystyrene microsphere calibration curves, however, fell within <3% of MV size measured by RPS. Thus, vesicle:microsphere SERs determined from platelets provide a valid method for estimation of MV diameter.
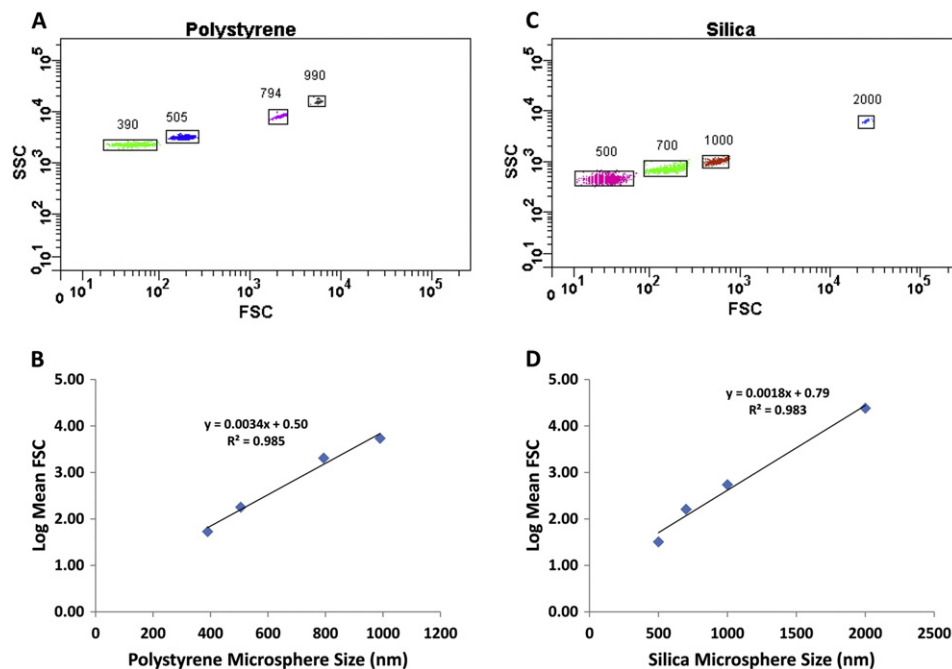
Fig. 1. **Polystyrene and silica microsphere size calibration.** See "Methods" for preparation of microsphere suspensions. Side scatter (SSC) vs. FSC dot plots of polystyrene and silica microspheres (A and C respectively). Size calibration curves for polystyrene and silica microvesicles (B and D respectively) were generated from log-transformed mean FSC values of microspheres versus microsphere size.
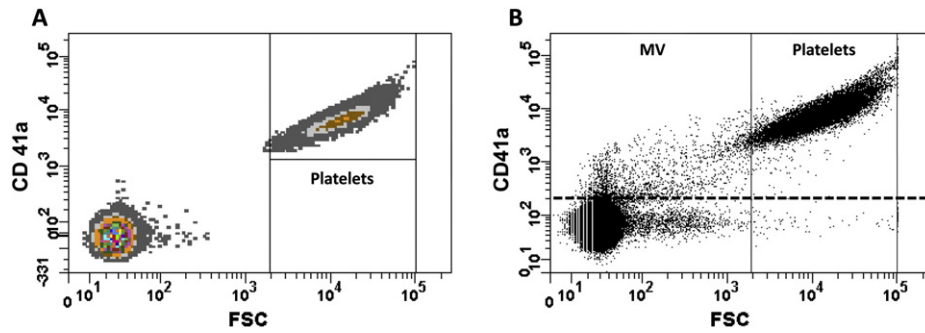
Fig. 2. Density and dot plots of PRP. (A) Density plot of anti-CD41a-APC-H7 fluorescence vs. FSC set at 2% probability. (B) Gates delineate CD41a+ platelet events. The dashed horizontal line represents the isotype fluorescence threshold. All events above the isotype line are positive for CD41a. All CD41a+ events to the left of the platelet gate are platelet-derived MV.

Fig. 5 shows SSC vs. FSC dot plots of a representative PRP sample superimposed with lines that identify the FSC of silica and polystyrene microspheres. Platelets in PRP were identified by anti-CD41a-APC-H7 fluorescence vs. FSC and the FSC gates were applied in SSC vs. FSC dot plots. Lines were placed at FSC values for 0.5-, 0.9-, and 1-μm microspheres as calculated from silica and polystyrene microsphere calibration curves. The FSC of all three sizes of silica microspheres fell below the lowest FSC gate of the platelet population while the FSC of 0.9-μm and 1-μm polystyrene microspheres fell within the platelet gates. Furthermore, FSC of 0.5-μm polystyrene microspheres was greater than FSC of many of the MV events. The percent contamination of MV by platelets was calculated as the number of events between 1-μm microspheres and the lower platelet gate ("PC") divided by the number of events between 1-μm microspheres and the resolution of FSC from noise (FSC = 10, "MV"). Use of polystyrene microsphere calibration curves to set the upper limit of the MV gate to 1 μm yielded a median platelet contamination of 16.53% (8.24%, 20.98%) within the MV population. Platelet contamination was not observed, however, when the 1-μm gate was set from the silica microsphere calibration curve.

Discussion

Flow cytometers detect scattered and fluorescent light emitted from particles irradiated from multiple lasers at several wavelengths. Scattered light is measured at different angles to the incident light, which is most often derived from a 488-nm laser. FSC detectors measure light scattered at ranges between 1° and 20° from the angle of the incident light depending on the instrument while SSC detectors measure light scattered at 90° from incident light. The intensity of

scattered light depends on multiple instrument and particle characteristics. These include wavelength and intensity (laser power) of irradiant light, angle of detection of scattered light, voltages and types of FSC and SSC detectors, size and shape of particles, and optical characteristics of particles e.g. refractive index and absorption [16,19]. Consequently, there are not absolute FSC and SSC intensities for all types of particles of different sizes. Cytometers, instead, must be calibrated with particles of known sizes and optical characteristics that are similar to particles of interest to obtain closest estimates of size.

Mie theory describes intensities of electromagnetic radiation scatter by objects at different angles to the incident light. It takes into account many properties that include wavelength and power of irradiant light, angle of scatter, particle shape, and particle refractive index (η). Chandler et al. applied Mie theory to estimate theoretical FSC intensities from polystyrene and silica microspheres and platelet MV irradiated with 488-nm light as a function of particle size and refractive index [19]. Mie calculations predicted that the relative order of FSC intensities for any given size follows refractive index of the particle: polystyrene ($\eta = 1.59$) FSC > silica ($\eta = 1.463$) FSC > platelet MV ($\eta = 1.39$) FSC. Measured FSC for polystyrene microspheres, silica microspheres, and platelet and liposome vesicles substantiated the predicted relative order.

In an effort to standardize quantification of MV by flow cytometry, the International Society on Thrombosis and Haemostasis Scientific Standardization Committee (ISTH-SSC) has recommended the use of fluorescent 0.5-μm and 0.9-μm polystyrene microspheres (Megamix microspheres; BioCytex; Marseille, France) to set the lower and upper gates, respectively, that define MV events [22]. Although use of these microspheres allows investigators to set the window of analysis reproducibly, because the optical properties of polystyrene microspheres
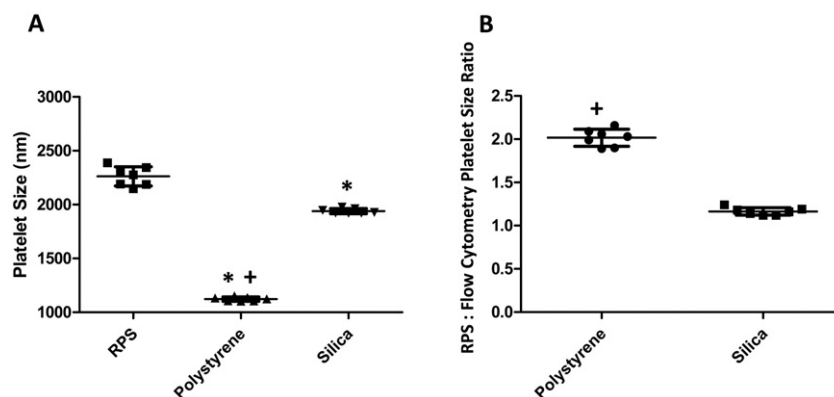
Fig. 3. Comparison of platelet size as determined by RPS and flow cytometry. (A) Platelet size in PRP from seven donors was measured by RPS and from FSC values using calibration curves generated from polystyrene and silica microspheres of different sizes. (B) The "RPS: Flow cytometry platelet size" ratios were calculated from platelet size measured by RPS divided by platelet size measured by flow cytometry calibrated with polystyrene and silica microspheres, and reflects the relationship between platelet size measured by flow cytometry and RPS (an estimation of actual platelet size). *Significantly different from "RPS" ($p < 0.001$). +Significantly different from "Silica" ($p < 0.001$).
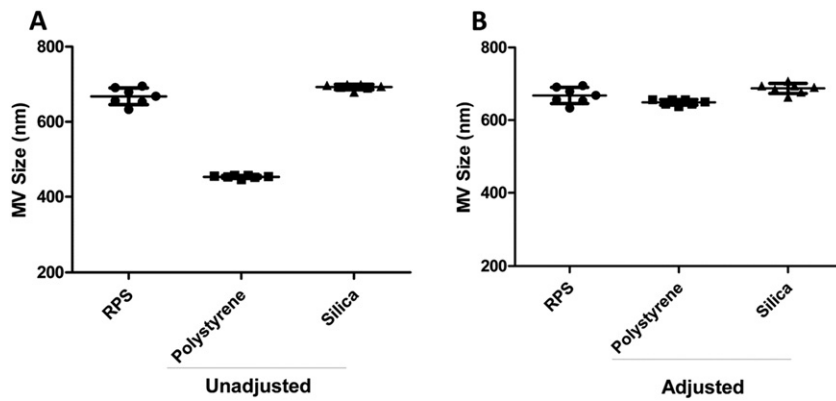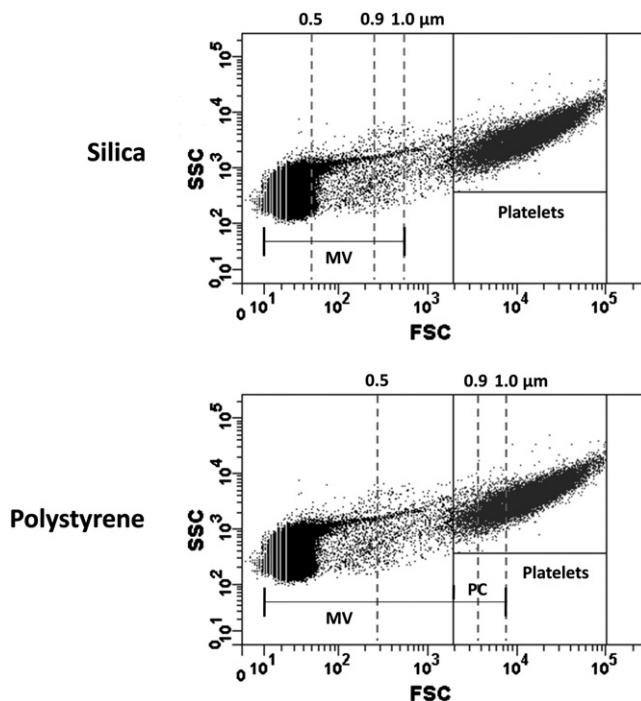
Fig. 4. Comparison of MV size determined by RPS and flow cytometry with and without adjustment for vesicle:microsphere size equivalency ratios. The mean diameter of MV in PPP between sizes 600 and 800 nm was measured by RPS and by flow cytometry and compared. (A) Unadjusted MV sizes determined by flow cytometry were calculated directly from the polystyrene and silica microsphere calibration equations after calculation of FSC gates for 600 nm and 800 nm from the silica microsphere calibration equation in Fig. 1. (B) Adjusted MV sizes were calculated from FSC gates that had been adjusted to 300 nm and 400 nm for polystyrene microspheres and to 500 nm and 667 nm for silica microspheres through division by vesicle:microsphere SERs of 2.0 and 1.2 for polystyrene and silica, respectively, (Fig. 3B) as described in "Results."

are vastly different from those of cellular MV the FSC intensities of 0.5-μm and 0.9-μm polystyrene microspheres are equivalent to FSC from much larger cellular vesicles. The exact FSC equivalencies depend upon instrument detector type (photomultiplier tube vs photodiode) and angle of detection (narrow [1°-8°] less sensitive to smaller MV vs wide [1°-19°]) as well as the electronics such as amplifiers and voltage settings [16,19]. Van der pol et al. [16] reported that FSC from 0.5-μm and 0.9-μm polystyrene microspheres corresponded to FSC of 1.0-μm and 1.75-μm vesicles, respectively, on a Beckman Coulter FC500 cytometer and corresponded to 1.25-μm and 2.0-μm vesicles, respectively, when

Fig. 5. Platelet contamination within MV gates. Platelets in PRP were identified by anti-CD41a-APC-H7 fluorescence and gated in SSC vs. FSC dot plots. The 0.5-μm, 0.9-μm, and 1.0-μm FSC values were calculated from the equations that describe standard curves generated from silica and polystyrene microspheres. The "MV" gates were designated from the respective 1-μm microsphere FSC upper size down to the limit of resolution from instrument noise. Platelet contamination ("PC") of the MV gate was calculated as the number of events between the 1.0-μm polystyrene microsphere and the lower platelet gate divided by the total events in the "MV" gate x 100. Median platelet contamination for seven PRP samples was 16.53% (8.24%, 20.98%).

detected on an Apogee A40. Chandler et al., however, calculated equivalent vesicle diameters of 1.4 μm and 2.7 μm for 0.5 μm and 0.9 μm Megamix microspheres, respectively, on an Apogee A40 cytometer (17, 19). Thus, vesicle:Megamix polystyrene microsphere SERs ranged from 1.9:1 to 3.0:1 depending on instrument. That is, the FSC of Megamix microspheres was equivalent to the FSC of vesicles 1.9 to 3.0 times larger, depending on the cytometer used. This would result in 1.9 to 3.0-fold underestimation of vesicle size.

In the present study, we observed a 2.0:1 vesicle:microsphere SER between polystyrene microspheres and platelets and a 1.2:1 vesicle:microsphere SER between silica microspheres and platelets (Fig. 3). Based on this data we estimate that gates set on our cytometer using polystyrene microspheres that are 0.5 μm and 0.9 μm in diameter as recommended by the ISTH-SSC actually selects for vesicles that are 1.0 μm and 1.8 μm, respectively. Hence, use of 0.5-μm and 0.9-μm poly-styrene microspheres to define MV events selects a population of vesi-cles outside the established MV range that includes platelet-sized vesicles. Setting the gates using 0.5-μm and 0.9-μm silica microspheres, however, selects for vesicles between 0.6 μm and 1.1 μm, which ex-cludes platelets (Fig. 5). The MV size data in Fig. 4 that was adjusted for the different vesicle:microsphere SERs validate these estimates and demonstrate that the relationship between the size of vesicles and mi-crospheres with respect to FSC is consistent over the MV and platelet size ranges.

Many laboratories continue to use 1-μm polystyrene microspheres to set the upper boundary and the limit of resolution of their instrument from noise as the lower boundary to define the MV events [17,18]. Fig. 5 illustrates the impact of the large discrepancy in FSC between polysty-rene microspheres and vesicles on the size of particles in a MV gate. The FSC of 0.9-μm and 1.0-μm polystyrene microspheres fell within the range of FSC values of platelets. As a result, we observed a median platelet contamination of 16.53% (8.24%, 20.98%) of the MV gate desig-nated from the 1-μm polystyrene microsphere FSC down to the noise limit of our cytometer. Platelet contamination was calculated as a percentage of the total MV events. Two of the seven PRP samples contained unusually high numbers of MV (15- and 173-times the average of the other five PRP samples), which resulted in apparently low platelet contamination values of 2.7% and 0.2%, respectively. The platelet numbers in those samples were, however, higher than in the other five samples. Excluding those samples, the median platelet contamination was 20.28% (16.53%, 21.68%) of the MV events. In contrast, the MV gate established from 1-μm silica microspheres did not contain platelets from any of the PRP samples. Contrary to our results, in a study by Robert et al. [23] platelets fell outside the 0.9 μm bead peak. This could be due to the fact that they used a FC500

cytometer and we used a BD FACS Canto II cytometer. In another study Mullier et al. [24] have reported minimal platelet contamination in the MV gate. In that study they used three different cytometers; FACS Aria, FACS Canto II and Accuri C6. The data shown in their Fig. 1 C shows some platelet contamination in the PMP gate. However; it is not as pronounced as that reported in the present study. This difference could be due to the methods used to define the platelet and PMP gates. Mullier et al. used 0.9 micron beads to define their platelet gate whereas we derived the FSC values for one micron from a standard curve using four point calibrations with 390, 505, 794 and 990 nm beads. As depicted in Fig. 1, our R^2 value for the standard curves was 0.98 indicating a linear relationship between bead size and FSC on our Canto II.

The normal size distribution of platelets is between 1.5 and 3 μm [25]. So, the accepted upper size limit that defines MV is designed to exclude platelets, the smallest of the blood-borne cellular elements. Our data and data from others showed that, depending on the instrument, setting the upper MV limit using 1-μm polystyrene microspheres includes all vesicles between 2 μm and 3 μm in plasma preparations [16, 19]. From our data, the FSC from 1.0-μm silica microspheres, however, is equivalent to the FSC of vesicles that are 1.2 μm in diameter on our instrument, which falls below the lower size of normal platelets (Fig. 5). Vesicle:polystyrene microsphere SERs reported in the literature have demonstrated instrument-dependence. It is likely then that SERs for silica microspheres will also vary by instrument. Investigators should, therefore, measure the vesicle:silica microsphere SER with platelets in PRP on their instrument to ensure that platelet-sized events are not included within the MV population defined by 1.0-μm silica microspheres and to adjust appropriately estimations of MV diameters. Platelet size in PRP can be measured conveniently in any hematology cell counter or Coulter Counter.

We compared platelet and MV diameters calculated from polystyrene and silica-derived calibration curves to diameters obtained from an independent method of size measurement (RPS) that is based on the well-established Coulter principle and showed that on average there was a significantly better correlation between RPS size and silica-based size determination than between RPS size and polystyrene-based size determination (Fig. 3). Though TEM is regarded as the gold standard technique to measure sizes of cells and cell MV, in a recent publication, Anderson et al. [26] demonstrated that among four different platforms for sizing submicron particles, "tunable resistive pulse sensing (TRPS) and differential centrifugal sedimentation (DCS) provided the greatest resolution and accuracy, being the only techniques to distinguish the peak maximum (modal) values of the 220, 330 and 410 nm sub-populations present within the multimodal sample to within 5% of the TEM reference measurements."

Recently, several reports have discussed the biological effects of MV related to their size [10–12,15,27]. Champion et al. demonstrated that phagocytosis of polymeric microspheres by macrophages is dependent upon the size of the microspheres [12]. Dean et al. found that different sizes of MV had different classes of proteins and have different functional effects on endothelial cells and platelets [15]. A recent study by Gardiner et al. demonstrates the role of different sizes of released MV as an indicator of embryo quality [27]. Size is an MV characteristic that has not been explored adequately. This is no doubt due in part to the challenges associated with measuring size of submicron vesicles by flow cytometry. A sizing protocol that yields accurate MV sizes reproducibly from laboratory to laboratory will contribute to our understanding of the relationship between MV size and biological effect. Use of silica microspheres to calibrate flow cytometers provides a close estimate of MV size.

We report here that use of silica microspheres to calibrate FSC to size provides significantly more accurate estimation of MV size than does the use of polystyrene microspheres and eliminates platelet-sized event contamination within the MV gate that occurs with use of 1-μm polystyrene microspheres. Although platelet contamination in the MV region is decreased in PPP, it will vary among donors and according to sample handling procedures. Furthermore, aggregates of MV that fall within the smaller platelet population will be included in MV defined by 1-μm polystyrene microspheres. The inclusion of platelet-sized event contamination within the MV gate can artificially increase the total and platelet MV concentrations that are reported, which could lead to erroneous conclusions about correlations between MV populations and physiologic events under study.

Addendum

Disclosure of Conflicts of Interest

The authors have no conflicts of interest.

Acknowledgements

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